Aster tataricus alleviates constipation by antagonizing the binding of acetylcholine to muscarinic receptor and inhibiting Ca^{2+} influx

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ABSTRACT

Background: The dried root and rhizome of *Aster tataricus* (RA), is a traditional Chinese medicine has been used for more than 2000 years with the function of antitussive, expectorant and antiasthmatic. Ancient books and modern pharmacological researches demonstrated that RA may have the function of moistening intestines and relieving constipation, but there was a lack of systematic evidence. The aim of this study was to comprehensively evaluate the efficacy and possible mechanisms of ethanol extract of *Aster tataricus* (ATE) in treating constipation from in vivo to in vitro.

Methods: In vivo, the ATE was studied in loperamide-induced constipation of mice. In vitro, different concentrations of ATE was tested separately or cumulatively on spontaneous and agonists-induced contractions of isolated rat duodenum strips.

Results: In vivo, at doses of 0.16, 0.8 g/mL, ATE showed significantly promotion of the small intestinal charcoal transit, decrease of the amount of remnant fecal, and increase of the content of fecal water in colon. In addition, ATE could effectively relieve colonic pathological damage caused by loperamide as well. In vitro, with the cumulative concentration increase of ATE from 0.8 to 6.4 mg/mL, it could significantly decrease the contraction caused by KCl or Ach, and gradually restore to near base tension value.Meanwhile, it could also partially but significantly inhibit the contractions induced by Ach and $CaCl_2$ on rat duodenum in a concentration related manner.

Conclusions: Taking all these findings together, it could be speculated that ATE may attenuate constipation mainly through antagonizing the binding of acetylcholine to muscarinic receptor, inhibiting Ca^{2+} influx and anti- inflammation.

1. Introduction

Constipation is a common disease of the digestive system. According to its etiology, constipation is divided into organic constipation and functional constipation. Functional constipation refers to chronic constipation without organic causes, structural abnormalities or metabolic disorders, except irritable bowel syndrome, whose main clinical symptoms are reduced number of defecation, dry fecal knot, defecation difficulty and defecation insufficiency [1]. Statistics showed that the prevalence of chronic constipation in foreign populations was 5%–20 %, while in China the prevalence was about 8.2 % [2–5]. Although constipation will not endanger our lives, long-term constipation will cause complications of anorectal diseases such as hemorrhoids and anal fissure, and increase the mortality rate of hypertension, cardiovascular and cerebrovascular diseases and the prevalence rate of colon cancer [6, 7]. The laxative drugs, such as Senna™, Correctol®, Senokot™, were mainly to assist patients in passing stools by promoting bowel movements [8]. However, these drugs had accompanying adverse side effects, including artery contraction, coronary spasms, and myocardial infarction [9–11]. Therefore, it is of great significance to find a new drug that can effectively treat constipation and possess less side effects.

Due to the complicated pathogenesis of constipation, the treatment methods of various drugs could also be different. Li et al. found that the laxative effects of Anemarrhena asphodeloides Bge. Polysaccharides were by enhancing the expressions of AQP3 and VIP, and decreasing the AQP4, AQP8 and PGE2 expressions [12]. Kim et al. found that Quercetin could treat constipation through regulating the expression of mAChRs and their downstream signals, as well as the secretion of mucin [13]. Zheng et al. found that the laxative effects of emodin were associated with the increased expression of AQP3 by up-regulating PKA/p-CREB signal pathway [14]. These findings provide good hints, but it is far from clear understand the exact mechanisms for treating constipation, so further studies are needed to determine the mechanisms of action of laxative drugs.

Aster tataricus L.f., which belongs to Compositae family, is a traditional Chinese herbal medicine with a long history that has been used in the treatment of coughs, phlegm and asthma [15,16]. The ancient books recorded that RA had the function of moistening intestines and relieving constipation, and other previous researches also indicated that water decoction of RA played a laxative role by regulating the content of neurotransmitters [17–19], but there were currently no systematic assessments of the laxative effects of RA and the mechanisms of its treatment were poorly understood. Therefore,

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the purpose of this study was to comprehensively evaluate the laxative effects of RA and its therapeutic mechanisms through the loperamide-induced constipation mice in vivo and the relaxation activity on rat duodenum in vitro.

2. Materials and methods

2.1. Drugs and reagents

NaCl, KCl, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, $NaHCO_3$, $NaH_2PO_4 \cdot 2H_2O$ and glucose were purchased from Beijing Huawei Ruike chemical Co. Ltd. (Beijing, China). Acetylcholine, atropine, and verapamil were purchased from Beijing Saipu Ruisi technology Co. Ltd.(Beijing, China). Maren Pills were purchased from Beijing Tongrentang (Beijing, China). EGTA, gum Arabic, and activated charcoal were purchased from Beijing Enokai technology Co. Ltd.(Beijing, China). Solutions of all the chemicals were dissolved in pure water.

2.2. Instrumentations

The BL-420 N signal acquisition and processing system is equipped with HW-500 super thermostatic water bath and SV-4 in vitro tissue and organ perfusion system. The above experimental instruments were purchased from Taimeng software Co., Ltd. (Chengdu, China).

2.3. Experimental animals

The animal protocol used in this study was reviewed and approved based on ethical procedures and scientific care by the Beijing University of Chinese Medicine-Institutional Animal Care and Use Committee (Approval Number, No.4− 2018010103-10,045). Adult KM mice (18− 22 g) and SD rats (180− 220 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. All the animals were housed under a constant temperature of 25 ± 2 °C, a relative humidity of 50 ± 10 %, and a 12 h light/dark cycle.

2.4. Plant material and preparation of ATE

The RA was purchased from Beijing Tongrentang and identified by Professor Xueyong Wang, the specimens (No. CMAT-AT-201,904) were placed in the laboratory of analysis and transformation of traditional Chinese medicine (Beijing University of Chinese Medicine). The ethanol extract of RA (1 kg) was obtained by the following process: 50 % ethanol, 1:10 of solid-liquid ratio, and 1.5 h of each reflux extraction for twice, then the extract was filtered and concentrated to a certain volume, stored in a refrigerator at − 80 °C [20].

2.5. In vivo study

2.5.1. Animal experimental design

Constipation was induced in the animals by orally administered 4 mg/kg (Body weight) of loperamide at 09:00 and at 18:00 for three days, while the control mice were administered pure water [21]. ATE was diluted in pure water to a final concentration of 0.032, 0.16 and 0.8

g/mL (10 mL/kg, Body weight) and orally administered once a day for 3 days 1 h after each loperamide administration as suggested by the previously reported studies. For positive drug group was treated with Maren Pills at a dose of 4 g/kg (Body weight). Then, test animals were fasted and drank only 18 h prior to the experiment. On the 4th day, at 9:00, the control group was given pure water, while the others were received 4 mg/kg of loperamide. 1 h later, all groups were fed with the prepared charcoal meal (Among them, the charcoal meal of Control group and Model group did not contain any drugs, while the charcoal meal of Maren Pills group and ATE group contained corresponding doses of drugs) [22].

2.5.2. Measurement of intestinal charcoal transit ratio

Intestinal charcoal transit was measured through prepared charcoal meal [23]. Thirty minutes after administration of the charcoal meal, these mice were sacrificed by necking and their small intestines were quickly removed from the abdominal cavity [24]. The intestinal charcoal transit ratio was calculated as follows:

Charcoal transit ratio (%) = (transit distance of charcoal meal/total intestine length) × 100 %

2.5.3. Measurement of remnant fecal numbers and water content in colon

After the measurement of intestinal charcoal transit ratios, we took out the whole colon. The total number and water content of fecal pellet remnants in the colon lumen were quantified individually. The fecal pellets water content was also analyzed as follows:

Fecal pellets water content (%) = (A − B)/A × 100 %

A is the weight of fresh fecal pellets collected from the colon, and B is the weight of fecal pellets after drying at 60 °C for 12 h [25].

2.5.4. Histopathological analysis

The taken colons were washed with phosphate buffered saline (PBS) (pH 7.4). After filling with 4% paraformaldehyde to the appropriate degree, the colons collected from all groups were fixed for 48 h. The samples were subsequently embedded in paraffin wax, after which they were cut into 4 μm thick sections and stained with hematoxylin and eosin (H&E). The sections were then analyzed for mucosa thickness, the integrity of the gland and flat luminal surface, and number of goblet cells in the colons by light microscopy and the Leica Application Suite.

2.6. In vitro study

2.6.1. Tissue preparation

SD rats were starved for food for 18 h but allowed free access to water. They were sacrificed by cervical dislocation and then 3–4 small segments of duodenum (1–1.5 cm long) were excised from close to the gastro-intestinal junction, placed in a cold oxygenated Tyrode's solution with the following composition in mM: NaCl 136.90, KCl 2.68, $MgSO_4 \cdot 7H_2O$ 1.05, $CaCl_2$ 1.80, $NaHCO_3$ 11.90, $NaH_2PO_4 \cdot 2H_2O$ 0.42, and glucose 5.55 and gently cleaned of fat and adherent connective tissues [26,27]. The strips were subsequently suspended in a 10 mL organ bath containing Tyrode's solution maintained at 37 ± 5 °C and continuously bubbled with mixture (5% CO_2 and 95 % O_2) to maintain the pH at 7.4 [28,29]. The lower end of the strip was fastened to a fixed hook at the bottom of the organ bath, and the upper end was connected with a steel hook to an isotonic transducer combined with a computerized data processing system (BL-420 N, Biological signal acquisition and processing system, Chengdu, China) for continuous recording of mechanical activity [30,31]. Prior to drugs administration, each strip

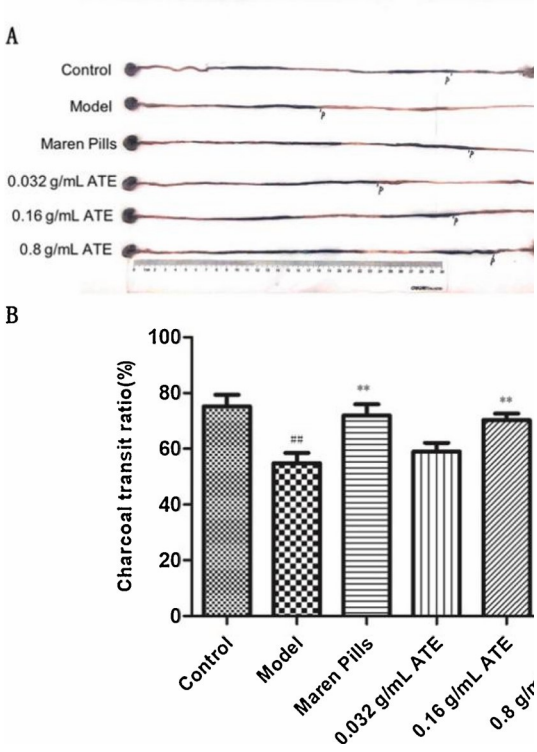

Fig. 1. (A) An actual image of the charcoal meal transit and the intestine. (B) Small intestinal transit rate of mice after constipation was induced by loperamide and treated with ATE. The data are reported as the mean ± SD (n = 6). ##P < 0.01 compared to the control group, while **P < 0.01 and ***P < 0.001 compared to model group.

was allowed to equilibrate in the Tyrode's solution for at least 60 min at a baseline tension of 1 g. During the equilibration period, the bathing solution was renewed every 15 min.

2.6.2. Measurement of the effects of the ATE on spontaneous contractions

After the equilibration, different concentrations of ATE (0.8, 1.6, 3.2, 4.8 and 6.4 mg/mL) were tested separately on rat duodenum spontaneous contractions according to the method described by Hu and Wang with some modified [32,33]. Each concentration was added to the bath solution and its effects were recorded for 10 min. Then observe the changes in the contraction curve.

2.6.3. Measurement of the effects of the ATE on KCl-induced contractions

KCl (20 mM) was applied to the bath solution to induce a sustained contraction [34]. Once the contraction was relatively stable, cumulative concentrations of ATE (0.8, 1.6, 3.2, 4.8 and 6.4 mg/ mL) were sequentially added every 5 min. Concentration-response curves for the ATE were constructed and the EC_{50} and E_{max} were determined. *2.6.4. Measurement of the effects of the ATE on Ach-induced contractions* In the first set of experiments, Ach (10^{-6} M) was applied to the bath solution to induce a sustained contraction. Once the contraction was relatively stable, cumulative concentrations of ATE (0.8, 1.6, 3.2, 4.8 and 6.4 mg/ mL) were sequentially added every 5 min. Concentration- response curves for the ATE were constructed and the EC_{50} and E_{max} were determined.

In the second set of experiments, after equilibration, increasing concentrations of Ach (10^{-9}-5 × 10^{-6} M) were cumulatively applied to the bath solution at 2 min interval and a reference concentration- response curve was recorded. Then, the strip was pretreated with a single concentration of ATE (0.8, 1.6 and 3.2 mg/mL) or positive drug (atropine, 1 μM) for 5 min and the concentration-response curve to Ach was re-examined in the continuous presence of the drug [35].

2.6.5. Measurement of the effects of the ATE on $CaCl_2$-induced contractions

To determine the effects of ATE on Ca^{2+} influx, the stripe was initially equilibrated in Tyrode's solution, and then Tyrode's solution was replaced with Ca^{2+}-free high-K^+ (60 mM) solution containing EDTA (0.1 mM) for 30 min in order to remove Ca^{2+} from the tissues [36]. After equilibration, increasing concentrations of $CaCl_2$ (10^{-4}-3 × 10^{-2} M) were cumulatively applied to the bath solution at 2 min interval and a reference concentration-response curve was recorded. Then, the strip was pretreated with a single concentration of ATE (0.8, 1.6 and 3.2 mg/mL) or positive drug (verapamil, 0.025 mM) for 5 min and the concentration-response curve to $CaCl_2$ was re-examined in the continuous presence of the drug [37,38].

Data were expressed as mean ± SD or percentage and analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Dunnett's test. Test was performed using GraphPad Prism package version 5.00 (GraphPad Software, San Diego, CA, USA). P- Value less than 0.05 was considered to be statistically significant.

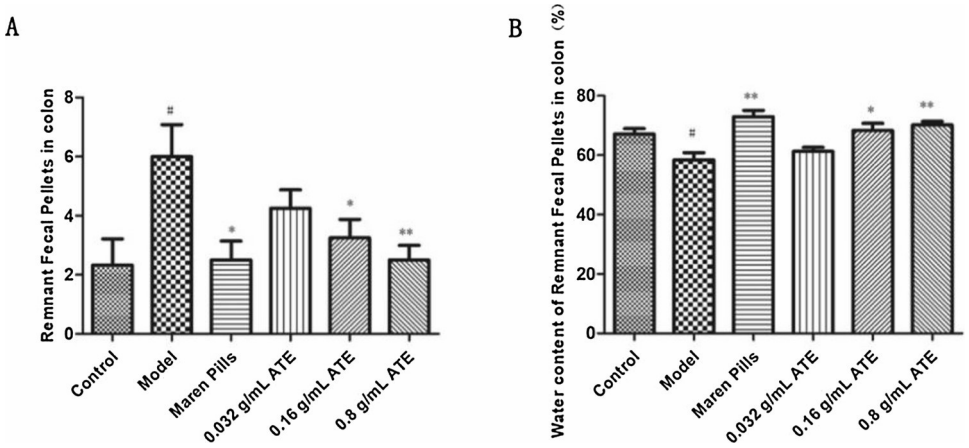

Fig. 2. (A) The numbers of remnant fecal pellets in colon. (B) The water content of remnant fecal pellets in colon. The data are reported as the mean ± SD (n = 6). #P < 0.05 compared to the control group, while *P < 0.05 and **P < 0.01 compared to model group.

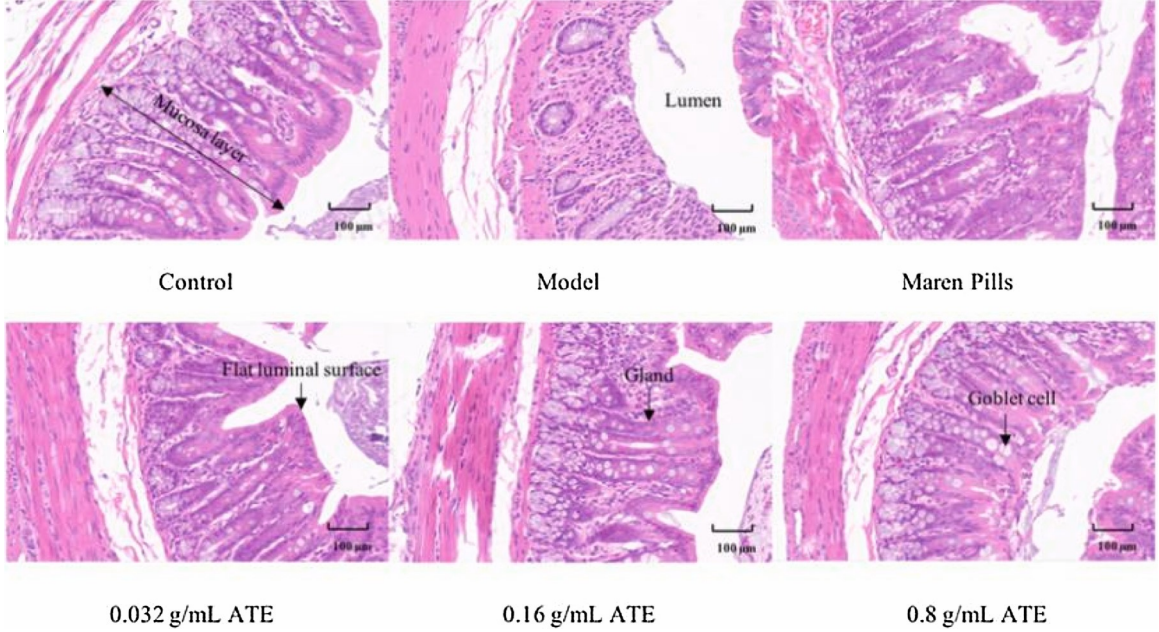

Fig. 3. Representative slides of H&E staining results for colon sections of the control group, model group, Maren Pills group and ATE (0.032, 0.16, 0.8 g/mL) group (magnification, × 200).

3. Results

3.1. Effects of ATE on small intestinal charcoal transit ratio

Small intestinal charcoal meal transit test is an important standard in constipation [39]. As shown in Fig. 1, the propulsion of the charcoal meal was significantly decreased 27.07 % in the Model group compared with Control group. After the administration of ATE and Maren Pills, compared with Model group, the charcoal transit ratio of 0.16 and 0.8 g/mL ATE groups were significantly increased 28.12 % and 52.65 % respectively, Maren Pills group was significant increased 31.14 %, while the 0.032 g/mL ATE group was no significant difference.

3.2. Effects of ATE on remnant fecal numbers and water content in colon

It could be seen from Fig. 2 A, the mice of Model group showed increase in remnant fecal numbers in the colon compared with the Control group. However, at sacrifice, compared with Model group, the remnant fecal number in the colon was decreased in mice treated with 4 g/kg Maren Pills, 0.16 and 0.8 g/mL ATE, while at dose of 0.032 g/mL ATE, was no significant difference. At the same time, we measured the water content in remnant fecal numbers in colon. As shown in Fig. 2 B, we observed that the water content in the Model group was decreased compared with the Control group, however, after the administration, the Maren Pills, 0.16 and 0.8 g/mL ATE groups were increased.

3.3. Effects of ATE on histological structure of the colon

As shown in Fig. 3, we observed that the flat luminal surface and the gland of the colon was damaged, the mucosa became thinner and the goblet cells were reduced in the model group. However, these damages could be significantly mitigated by administration of ATE or Maren Pills, but the levels did not reach those of the Control group. Taken together, these findings showed that ATE contributed to the recovery of abnormalities observed in the colon of loperamide-induced constipated mice.

3.4. Effects of ATE on spontaneous contractions

The representative actual contraction curve illustrated the effects of

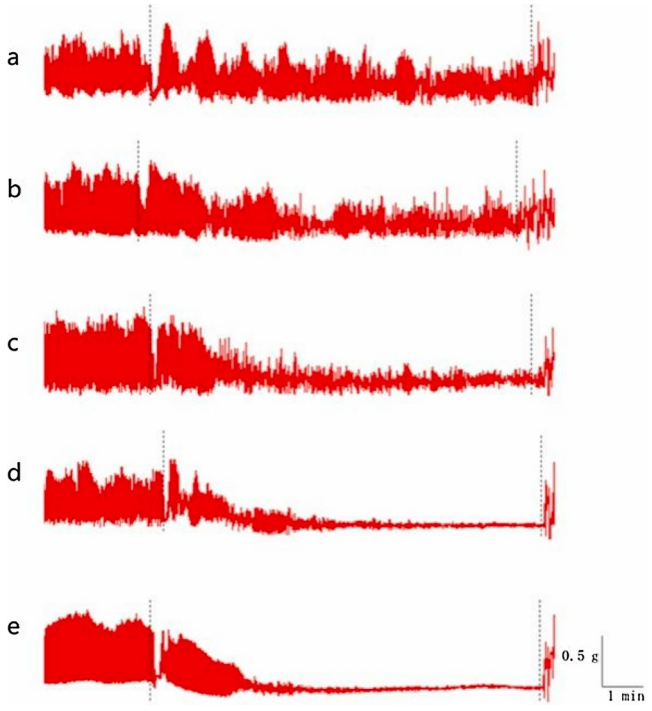

Fig. 4. An actual contraction curve of different concentrations of ATE on spontaneous contraction in isolated rat duodenum. 0.8 mg/mL; b 1.6 mg/mL; c 3.2 mg/mL; d 4.8 mg/mL; e 6.4 mg/mL. A single concentration of ATE was administered in the bath solution and its effects were observed for 10 min on the spontaneous contractions.

single concentrations of ATE on spontaneous contractions of rat duodenum were shown in Fig. 4. As shown in Fig. 5, the ATE (0.8, 1.6, 3.2, 4.8,

6.4 mg/mL) elicited an inhibitory effect on spontaneous contractions of rat duodenum, by reducing both the tension and the amplitude of contractions in a concentration-dependent manner. When the concentration of ATE reached 6.4 mg/mL, it reached a maximal level (E_{max}), and the contraction tension and contraction amplitude decreased 30.67

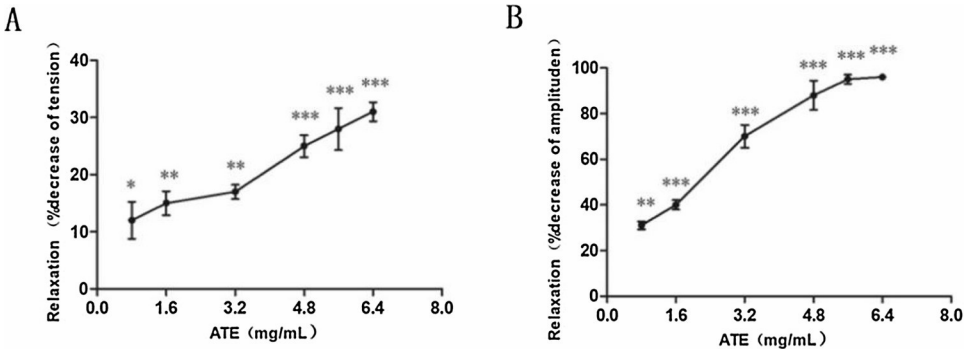

Fig. 5. Concentration–response curves of the relaxant effect of ATE on the tension (A) and the amplitude (B) of spontaneous contractions in isolated rat duodenum. The different concentrations of ATE was administered in the bath solution and its effects were observed for 10 min on the spontaneous contractions respectively. Data are expressed as mean ± SD of percentages of decrease of the tonus and the amplitude in ATE 0.8-6.4 mg/mL (n = 4) treated preparations. *P < 0.05, **P < 0.01, ***P < 0.001 compared to the to the initial tension and amplitude value.

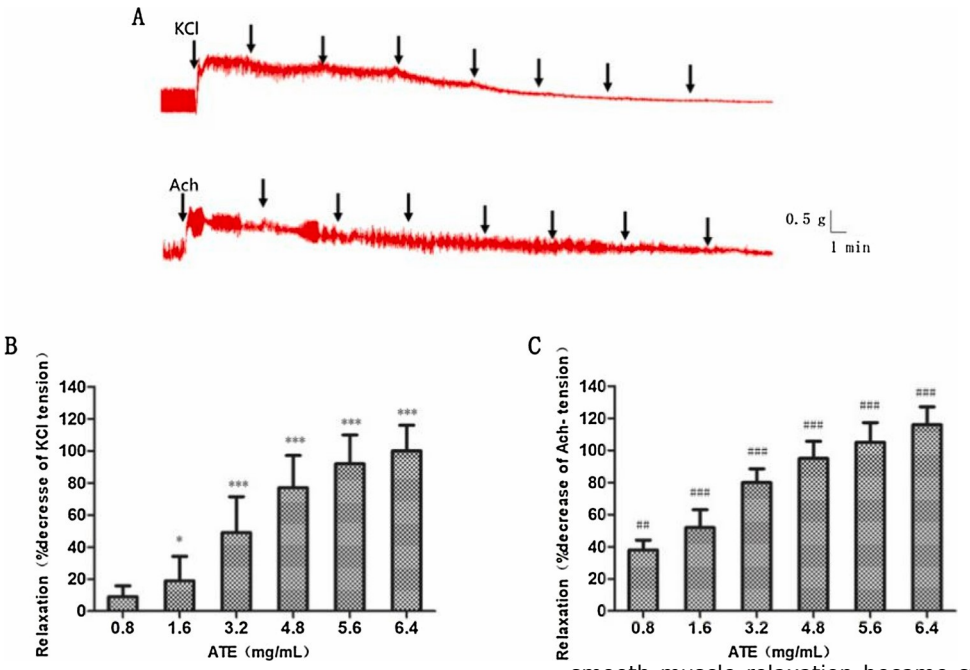

Fig. 6. Original tracing of the relaxant effect of ATE on KCl-induced and Ach-induced contractions in isolated rat duodenum (A). Bar graph of Ach (B). Bar graph of KCl (C). KCl (20 mM) or Ach (10^{-6} M) was applied to the bath solution to induce a substained contraction and ATE (0.8- 6.4 mg/mL) was cumulatively administered at 5 min intervals. The contractions induced by 20 mM KCl and 10^{-6} M Ach were regarded as 100 %. Data are expressed as mean ± SD of percentages of decrease of the tension in ATE 0.8-6.4 mg/mL treated preparations (n = 4). *P < 0.05, ***P < 0.001 compared to the KCl-induced tension value, ##P < 0.01, ###P < 0.001 compared to the Ach-induced tension value.

± 1.70 % and 96.05 ± 1.24 % respectively.

3.5. Effects of the ATE on KCl-induced contractions

The added value of tension of 20 mM KCl-induced contractions was taken as the base value and calculated as 100 %, then the change rate of tension after cumulative administration of ATE was calculated. It could be seen from Fig. 6 that the ATE could reduce KCl-induced contractions in a dose-dependent manner. When the concentration of 6.4 mg/mL of ATE, the maximum inhibition ratio was reached, and the value was 100.67 ± 16.06 %. The EC_{50} of ATE on the tension of KCl-induced contractions was 5.00 mg/mL.

3.6. Effects of the ATE on Ach-induced contractions

In the first set of experiments, the added value of tension of 10^{-6} M Ach-induced contractions was taken as the base value and calculated as 100 %, then the change rate of tension after cumulative administration of ATE was calculated. As shown in Fig. 6, with the concentration of ATE increased, its effect on smooth muscle relaxation became stronger and stronger. When the concentration of 6.4 mg/mL of ATE, the maximum inhibition ratio was reached, and the value was 115.67 ± 11.09 %. The EC_{50} of ATE on the tension of Ach-induced contractions was 3.67 mg/ mL.

In the second set of experiments, Ach (10^{-9}-5 × 10^{-6} M) was administered cumulatively induced a concentration-dependent contraction on rat duodenum in the absence or in the presence of ATE and atropine. With the concentration of Ach increased, the tension was gradually increased and reached a maximal level approximately at 5 × 10^{-6} M of Ach, the added value of tension of 5 × 10^{-6} M Ach-induced contractions was taken as 100 %. Then, at the concentrations of 0.8, 1.6, and 3.2 mg/mL of ATE, the maximum percentage of increased intestinal contractions tension induced by Ach

were 58.50 ± 4.50 %, 48.25 ± 9.90 %, and 17.01 ± 0.05 % respectively. Just as atropine (1 μM), a M receptor antagonist, reduced the contraction to 5.50 ± 1.50 % (Fig. 7).

3.7. Effects of the ATE on $CaCl_2$-induced contractions $CaCl_2$ (10^{-4}-3 × 10^{-2} M) was administered cumulatively induced a concentration-dependent contraction on rat duodenum in the absence or in the presence of ATE and verapamil. The added value of tension of 3 × 10^{-2} M $CaCl_2$-induced contractions was taken as 100 % in the absence of drugs. It was observed from Fig. 8 that ATE (1.6, 3.2, 4.8 mg/mL) antagonized incompetitively the contraction of isolated duodenum strips of rats induced by cumulative concentration of $CaCl_2$, and the effect increased with the dosage increasing. Just as verapamil, a well- known calcium antagonist, the ATE (1.6, 3.2, 4.8 mg/mL) shifted the

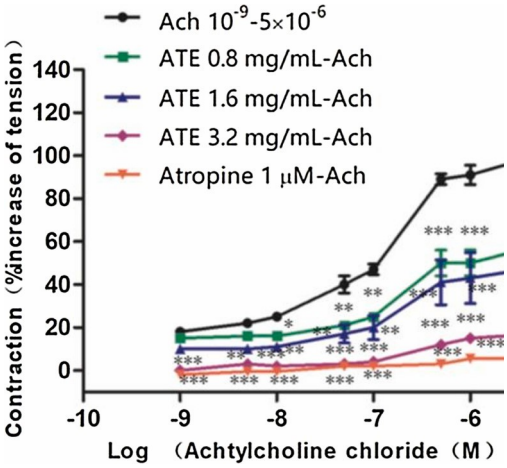

Fig. 7. Concentration–response curves of Ach on the isolated rat duodenum in the absence or in the presence of ATE and atropine. Ach (10^{-9}-5 × 10^{-6} M) was cumulatively applied to the bath solution and concentration–response curves were recorded in the absence or in the presence of ATE and atropine. Contractile response was expressed as a percentage of the maximal contraction (maximal tension) achieved in the absence of ATE or atropine. Data are expressed as mean ± SD of percentages of increase of the basal tension (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 compared to the absence of ATE or atropine.

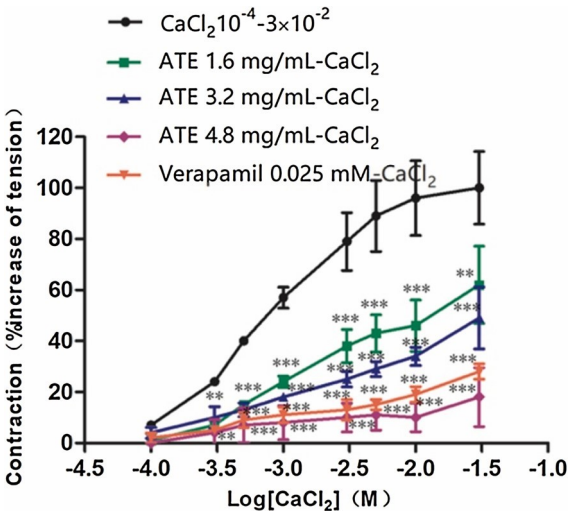

Fig. 8. Concentration–response curves of $CaCl_2$ on the isolated rat duodenum in the absence or in the presence of ATE and verapamil. $CaCl_2$ (10^{-4}-3×10^{-2} M) was cumulatively applied to the bath solution and concentration–response curves were recorded in the absence or in the presence of ATE and verapamil. Contractile response was expressed as a percentage of the maximal contraction (maximal tension) achieved in the absence of ATE or verapamil. Data are expressed as mean ± SD of percentages of increase of the basal tension (n = 4). **P < 0.01, ***P < 0.001 compared to the absence of ATE or verapamil.

$CaCl_2$ curves to the right and reduced the maximum contractions induced by 3 × 10^{-2} M $CaCl_2$ to 61.50 ± 15.19 %, 49.00 ± 12.19 % and 18.99 ± 11.60 % respectively, and verapamil (0.025 mM) reduced the contraction to 28.34 ± 3.01 %.

4. Discussion

Constipation is one of the most common gastrointestinal diseases in the world, especially among the elderly and causes too many problems. Although a large number of literatures indicated that constipation might be related to colonic motor dysfunctions, gastrointestinal hormone and neurotransmitter disorders, and imbalance of water metabolism, the exact pathogenesis of constipation was still unclear [40]. The results of the current study clearly demonstrated that ATE induced laxative effects, including the elevation of stool excretion and fecal water content in vivo, and inhibiting contractions of intestinal smooth muscles in vitro. Furthermore, our experimental results were the first to testify that ATE acted as calcium channels blocker and receptors blocker in the treatment of constipation.

An antidiarrheal drug, loperamide works by inhibiting water secretion and smooth muscle movement of the intestinal wall to prolong the excretion time and delay intestinal luminal transit [41–43]. Due to remarkable effects, it is widely used as a drug for constipation model by administering 4 mg/kg body weight of loperamide for 3–5 days in experimental animals [11–13]. These changes were also observed in

the loperamide-treated model mice used in our study. And after administrated with 0.16 and 0.8 g/mL ATE, a significant increase of charcoal meal transit was observed, which was consistent with the Maren Pills group exhibited similar inhibitory effects on the loperamide-induced decreases in intestinal motility. The result indirectly indicated that ATE had laxative effects against loperamide-induced constipation.

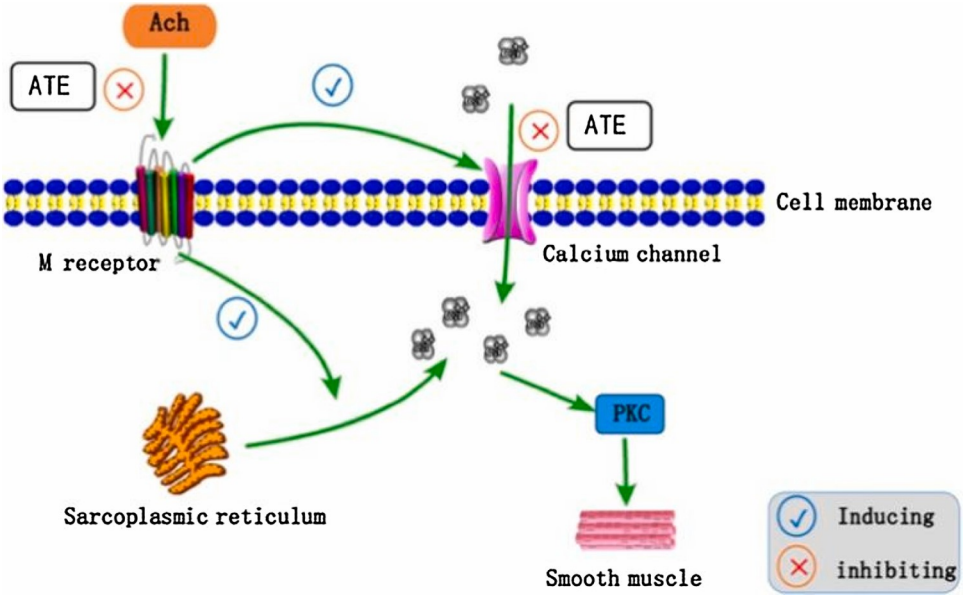

Fig. 9. The possible molecular mechanisms of the laxative effects of ATE.

Stool excretion is also considered as an important factor during the developmental and mechanistic study of laxative drugs, specifically, the number of defecations was significantly increased, the residual amount of feces in the large intestinal lumen was reduced and the water content of the feces was increased [44,45]. The loperamide could increase the remnant fecal numbers and reduce the water content in colon of constipation mice. These alterations dramatically recovered with the administration of drugs. In this study, a similar laxative effect on fecal-related factors was observed in the loperamide-induced constipation model treated with 0.16 or 0.8 g/mL of ATE, providing support that ATE has promising laxative effects at these doses. Our results also showed significant increases in the fecal water and decreases in the fecal numbers in the colon treated with Maren Pills.

Furthermore, histological studies have demonstrated significant alterations in the intestines of constipation-induced animals. The flat luminal surface and the gland of the colon was damaged, the thickness of the mucus layer is thinner and the goblet cells were reduced in the loperamide-treated groups than the vehicle-treated groups. However, these alterations recovered completely after treatment with several natural products, including AEtLP and AABP [11,12]. As presented in Fig. 3, the histological alterations of the colons observed in the present study were very similar to those of previous studies reported by Kim et al. [11,12].

The effect of laxative drugs is essential to the movement of intestinal smooth muscles, and intestinal smooth muscles are a spontaneous rhythmic contractive activity, with associated slow waves or spike potentials, is initiated by interstitial cells of Cajal distributed in the myenteric region [46–48]. However, the generation and conduction of spontaneous activities of Cajal mesenchymal cells are susceptible to the effects of ionic environment, neurotransmitters, hormones and other bioactive substances [49]. So, it is necessary to extend the study to its effects on intestinal contractions for the purpose of elucidating the mechanism of laxative activity. In the present study, decline of the tonus and amplitude of spontaneous contractions induced by ATE might be by regulating the release of neurotransmitters or by blocking the influx of calcium into the muscle cell through voltage-gated Ca^{2+} channels [50–52].

Moreover, the laxative activity of ATE could be either produced as a result of a non-specific membrane mediated effect or a more specific receptor mediated effect [53]. The release of the neurotransmitter Ach activates muscarinic receptors on smooth muscle cells, which in turn stimulate spontaneous muscle contractions [32,54]. In our study, we investigated the effects of single and cumulative concentration of ATE on Ach-induced duodenal contraction in rats. The experimental results indicated that ATE significantly inhibited the contractility of isolated tissue preparations of rat duodenum treated with Ach. As shown in Fig. 9, these results proved that the Ach directly activated their specific receptor on the membrane of gastrointestinal smooth muscle cells [55], which resulted in activation of several intracellular pathways and production of second messengers which in turn stimulated the release of internal Ca^{2+} from sarcoplasmic reticulum stores and the Ca^{2+} influx through voltage-gated Ca^{2+} channels

[56–58]. Therefore, the inhibitory effects of ATE on Ach-induced contractions was caused by the blocking muscarinic receptors on the cell membrane.

In addition to neural factors, other studies indicated that relaxed smooth muscle components present in medicinal plants are usually mediated by calcium channels blockade [59]. K^+ is known to cause smooth muscle contractions through the opening of voltage-dependent Ca^{2+} channels, thus allowing an influx of extracellular Ca^{2+} causing a contractile effect and substances causing inhibition of K^+-induced contraction are considered to be blockers of calcium influx [60]. In our research, the experimental results showed that ATE inhibited contractile response induced by single concentrations of KCl in rat duodenum, which was consistent with previously reported findings.

At the same time, to further assess whether the laxative activity of ATE works based on calcium channels blockade, $CaCl_2$ was administered cumulatively induced a concentration-dependent contraction on rat duodenum in the absence or in the presence of ATE and verapamil. The results showed that the ATE shifted the $CaCl_2$ dose-effect curves to the right and down in a non-competitive manner, similar to that caused by verapamil, a standard calcium channel antagonist [61], providing evidence that ATE acted as a calcium channels blocker (Fig. 9).

Actullay, with the aid of UHPLC-Q TOF-MS/MS, a total of 32 compounds were identified, including 14 peptides, 8 organic acids, 4 flavonoids, 3 coumarins and 3 terpenoids. Among them, flavonoids, such as quercetin, could effectively improve chronic constipation induced by loperamide treatment in animal models [13]. Peptides, such as Astin C, had potential use in the treatment of colonic inflammation [62]. Meanwhile, organic acids (such as chlorogenic acid), coumarins (such as scopoletin) and terpenoids (such as shionone) in RA may also played an important role in inhibiting intestinal spasm and alleviating intestinal inflammation [63–65]. These studies could provide good hints for improving the understand of ATE in regulating constipation. However, whether and how these components contribute to the laxative effects of ATE, as well as their synergistic ways, remains to be further investigated. And these results could be found in the supplementary material (Fig. S1 and Table S1).

5. Conclusion

In vivo results exhibited that ATE administration induced the recovery of gastrointestinal transit, remnant fecal numbers, water content and histopathology in loperamide-induced constipation model. In vitro results demonstrated that ATE could significantly decrease the contraction caused by KCl or Ach, and gradually restore to near base tension value. Taking all these findings together, it could be inferred that ATE could distinctly attenuate constipation mainly through antagonizing the binding of acetylcholine to muscarinic receptor, inhibiting Ca^{2+} influx and anti-inflammation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationship that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.111005 .

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